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Chimeric Viruses Expressing Primary Envelope Glycoproteins of Human Immunodeficiency Virus Type I Show Increased Sensitivity to Neutralization by Human Sera

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We constructed a number of HXB2 viruses chimeric for the gp120 glycoprotein derived from a number of viable molecular clones obtained from a primary isolate. Comparative biological characterization of the parental primary viruses with the gp120.HXB2 chimeras demonstrated identical patterns of cell tropism and cytopathicity. Furthermore, both parental and chimeric viruses were insensitive to neutralization by sCD4 and a panel of conformation-dependent monoclonal antibodies, demonstrating that transfer of the gp120 protein alone was sufficient to confer a ''neutralization-resistant'' phenotype to the T-cell-adapted clone HXB2. We assessed the contribution of gp120 epitopes to the neutralizing immune response by comparing the sensitivity of these viruses to neutralization by a panel of sera from HIV-infected individuals. Seven of eleven sera tested were able to neutralize HXB2 and two or more of the chimeric viruses; in contrast, only one serum neutralized more than one of the parental primary virus clones. The association of gp120–gp41 envelope at the surface of infected PBMC cultures was measured in the presence or absence of soluble CD4. No differences in CD4-induced gp120 dissociation were seen between the chimeric and parental virus-infected cultures. Since gp120 conformation appeared the same between primary and chimeric viruses, we suggest that the ability of human sera to neutralize the chimeric viruses may be mediated by epitopes within gp41. © 1996 Academic Press, Inc.

INTRODUCTION

The majority of functional studies of the HIV-1 envelope glycoprotein have been carried out with laboratory-adapted isolates which have been extensively propagated in T-cell lines. However, it is now apparent that "primary" viruses, isolated directly from patient tissue and only allowed to replicate for one to two passages in peripheral blood mononuclear cells (PBMC) and/or macrophages, differ from laboratory adapted isolates in a number of important aspects. Some primary viruses replicate slowly and fail to induce a multinucleated cytopathic effect (termed slow/low and non-syncytial inducing, NSI) whereas others replicate with faster kinetics and are able to induce syncytia in their target cells (termed fast/high and syncytial inducing, SI) (Åsjö *et al.,* 1986; Cheng-Mayer *et al.,* 1988; Fenyö *et al.,* 1988; Tersmette *et al.,* 1989). Generally, NSI viruses fail to replicate in established cell lines, whereas SI viruses replicate in a variety of T-lymphoid and monocytoid cell lines. Several authors have reported that viruses isolated from the majority of infected individuals during the course of disease progression change their *in vitro* properties from NSI to SI phenotype, suggesting that the appearance of SI virus is associated with more rapid CD4 cell decline and onset of symptoms (Cheng-Mayer *et al.,* 1988; Tersmette *et al.,* 1989; von Gegerfelt *et al.,* 1991; Schuitemaker *et al.,* 1992; Connor and Ho, 1994).

Primary viruses are insensitive to neutralization by soluble CD4 (sCD4). Several reports have suggested that this resistance is mediated by a reduced affinity of the oligomeric envelope complex for sCD4 (Ashkenazi *et al.,* 1991; Moore *et al.,* 1992; Turner *et al.,* 1992). Recent data suggest that primary viruses are resistant to neutralization by the majority of monoclonal antibodies (mAbs) cloned from animals immunized with recombinant gp120. Furthermore, sera from individuals immunized with recombinant gp120 were generally unable to neutralize primary viruses (Moore *et al.,* 1995; Wrin *et al.,* 1995). This resistance to neutralization both by sCD4 and mAbs may reflect a structural motif common to all primary viruses. However, short-term culture of primary viruses in PBMC is likely to maintain high levels of variation and, in contrast to long-term propagation in clonal T-cell lines, such antigenic variation may be an important factor contributing to the apparent resistance of primary viruses to neutralization by various ligands (Hammond *et al.,* 1996). However, some primary viruses are sensitive to neutralization by a limited number of human mAbs and polyclonal human sera (Burton *et al.,* 1994; Conley *et al.,* 1994; Kattinger, 1994; Kostrikis *et al.,* 1996; Mascola *et al.,* 1994; Moore *et al.,* 1996; Scarlatti *et al.,* 1993;

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Trkola *et al.,* 1996; Weber *et al.,* 1995). It is interesting to note that there is little or no correlation between genotype and neutralization type in that some viruses are sensitive to neutralization by serum from individuals infected with viruses of diverse clades. Likewise, some individuals, including those belonging to "nonprogressor" cohorts (Cao *et al.,* 1995; Pantaleo *et al.,* 1995), have an antibody response that can neutralize many primary viruses. These results clearly indicate that antibodies capable of neutralizing divergent primary viruses exist in response to a natural infection. The question remains as to whether such antibodies can be generated by a recombinant antigen. At present, the epitopes recognized by such cross-neutralizing antibodies are not known; it is important that future studies be targeted at understanding the nature of such antibodies. Studies to define neutralization epitopes on primary viruses have mostly used human mAbs, the majority of which have failed to neutralize, despite being able to completely neutralize laboratory strains of HIV in T-cell-based assays (Sullivan *et al.,* 1995).

The envelope glycoprotein gp120/gp41 plays an important role in determining cellular attachment, entry, cytopathicity, and sensitivity to neutralization by sCD4 and mAbs. We have constructed a number of HXB2 viruses chimeric for the gp120 glycoprotein derived from a number of viable molecular clones obtained from a primary isolate (Fredriksson *et al.,* 1991; Tan *et al.,* 1993). Comparative biological characterization of the parental primary viruses with the gp120.HXB2 chimeras demonstrated identical patterns of cell tropism and cytopathicity. Furthermore, both the parental and chimeric viruses were insensitive to neutralization by sCD4 and a panel of gp120-specific mAbs, demonstrating that transfer of the gp120 protein alone was sufficient to confer a "neutralization-resistant" phenotype to the T-cell-adapted clone HXB2. In contrast, the chimeric viruses were as sensitive as HXB2 to neutralization by a panel of polyclonal human sera, suggesting that the observed neutralization was independent of gp120. Alternatively, association of primary virus gp120 with HXB2 gp41 transmembrane protein may result in an altered gp120/gp41 conformation(s) affecting the presentation of epitopes to human serum antibodies. However, we were unable to demonstrate any differences in gp120 conformation between primary and chimeric virus-infected cultures. These data are consistent with the interpretation that cross-neutralizing antibodies present in human sera may be targeted toward conserved epitope(s) located in gp41 (Muster *et al.,* 1993; Buchacher *et al.,* 1994).

MATERIALS AND METHODS

PCR amplification, cloning, and sequencing of gp120 and gp160 sequences

The PCR followed the protocols previously reported (McKeating *et al.,* 1993a). The gp120-encoding region was amplified from a panel of λ clones containing full-length HIV genomes (Frederiksson *et al.,* 1991) using the primers 626 (sense, restriction enzyme site underlined) 5′-GTG GGT CAC CGT CTA TTA TTG GG and 524 (antisense) 5′-CAC CAC GCG TCT CTT TGC CTT GGT GGG using 30 cycles of amplification (94°, 40 sec; 50°, 35 sec; 72°, 210 sec) in a volume of 50 μl with template containing 10 ng of proviral DNA and 16 ng of each primer, 200 *μM* concentrations of each deoxynucleotide triphosphate, 1× PFU buffer, and 0.5 units of PFU polymerase (Stratagene). The gp160-encoding region was amplified using the primers 626 and 945 (antisense) 5′ GGT CTC GAG GAG ATA CTG CTC CC using the same reaction conditions. To prevent exonucleolytic degradation the PCR product was frozen immediately upon completion of the reaction. Ten microliters of the PCR reaction was visualized on an agarose gel and PCR products were purified by the Geneclean procedure (Bio 101, La Jolla, CA), restriction digested with either *Bst*EII/*Mlu*I or *Bst*EII/*Xho*I, and ligated into pHXB2-MCS Δ env (McKeating *et al.,* 1993a). Ligation mixtures were transformed into competent *Escherichia coli* (TG2, New England Biolabs) and individual colonies screened for inserts by PCR using primers 626/524. Plasmid preparations of clones containing inserts were prepared using Wizard miniprep kits (Promega). The gp120 region of each of the λ clones was sequenced with a series of primers using a cycle sequencing protocol and an Applied Biosystems 373A automated sequencer (Arnold *et al.,* 1995).

Transfection of λ and HXB2 chimeric clones and biological characterization of recovered virus

All clones (5 μg of λ and 2 μg of pHXB2) were transfected into both HeLa and HeLa–CD4 cells using lipofectamine (Gibco BRL). In addition pHXB2 was also transfected into the H9 cell line. After 72 hr the transfected HeLa cells were cocultured with two million phytohemaglutinin (PHA)-stimulated PBMC for 24 hr. Transfected H9 cells were propagated in RPMI/10% FCS and cell-free supernatant was collected after 7 days. PBMC were recovered from the HeLa monolayer, washed, and cultured in RPMI/10% FCS/IL-2 (5 u/ml) medium for 21 days with the addition of fresh uninfected PBMC after 7 and 14 days. The extracellular fluid was tested for the presence of soluble p24 antigen as described previously (Sundqvist *et al.,* 1989; McKeating *et al.,* 1993a). The transfected HeLa–CD4 cells were monitored for their production of soluble and intracellular p24 antigen. Cells were fixed with methanol:acetone (1:1 ratio, stored at −20°) and incubated with mAbs specific for p24 (MRC ADP repository); bound antibodies were detected with a β-galactosidase-conjugated anti-mouse Ig and X-gal as described previously (Clapham *et al.,* 1992). Cells expressing p24 antigen give rise to colored foci, and those viruses capable of inducing cytopathic

effects in the HeLa–CD4 cells resulted in multinucleated foci which could be quantified. Both extracellular virus and infected PBMC were tested for their ability to initiate infection with a panel of T-cell lines, including MT2, SupT1, CEM, and U937 cells (Fenyö *et al.,* 1988; Boucher *et al.,* 1992). T-cell lines were monitored for signs of cytopathic effect and for the production of p24 antigen.

Cell-free supernatants were collected from PBMC cultures and assessed for the levels of infectious virus by determining the 50% infectious dose (ID_{50}) for PBMC. Infection was measured by the detection of p24 antigen (Sundqvist *et al.,* 1989; McKeating *et al.,* 1993a). ID_{50} values were determined by the Kaerber formula.

Neutralization assay

Neutralization was assessed using PHA-stimulated PBMC as target cells with determination of p24 antigen production as the endpoint as reported previously (von Gegerfelt *et al.,* 1991; Scarlatti *et al.,* 1993; Albert *et al.,* 1990). A panel of sera were chosen at random from asymptomatic HIV-1-infected individuals. Serum or mAb dilutions, 75 μl, were incubated at 37° (1 hr) with an equal volume of virus (in duplicate using three different dilutions, 1:5, 1:25, and 1:125). Following the virus/antibody incubation 100,000 PBMC in a final volume of 75 μl RPMI, 10% FCS, IL-2 (5 u/ml), mixed from two blood donors, were added. PBMC were washed on Days 1 and 3, such that cells were pelleted by centrifugation and the medium was changed (Albert *et al.,* 1993). The ID_{50} of the virus stock was determined in parallel and the neutralization assay was evaluated for virus dilutions containing 10–75 ID_{50}. Neutralization was defined as the ability of a serum to completely inhibit virus replication as assessed by p24 antigen production, that is, an absorbance value below the cutoff value for the antigen ELISA of 100 pg.

Gp120 quantification and ligand binding studies: ELISA

Extracellualr media from infected cultures were inactivated with 1% NP-40, which does not irreversibly denature gp120/gp160 (McKeating *et al.,* 1993a), and the concentration of gp120 present was determined by ELISA, as previously described using recombinant gp120 as a reference standard (Moore *et al.,* 1992). For ligand binding studies, detergent-solubilized gp120 was allowed to bind to the solid phase via the polyclonal antiserum D7324 (Aalto Bioreagents, Dublin, Ireland) at an input concentration of 50 ng/ml. The ability of mAbs and sCD4 to bind to the captured gp120 was assessed by previously published methods (Moore *et al.,* 1990).

RESULTS

Sequence analysis of KI4803 λ clones

A λ library was constructed from the genomic DNA obtained from a short-term PBMC culture (7 days) of HIV-1 isolate KI4803 (Frederiksson *et al.,* 1991). Eight full-length molecular clones were obtained, seven of which were shown to give rise to infectious virus after transfection into PBMC. Previously, we have reported phenotypic variation among the seven clones (Tan *et al.,* 1993) and were therefore interested in determining the level of envelope variation present. The gp120-encoding region was sequenced for each clone, and the amino acid translation of these sequences is shown in Fig. 1. The average pairwise distance between the clones was 3.4% (range, 1.6–5.4%) with an average Dn/Ds ratio of 1.51 (range, 0.62–2.21) (Nei and Gojobori, 1986). The majority of amino acid substitutions was conservative and was found in both the previously defined variable and the conserved regions (Modrow *et al.,* 1987). The V2 and V3–C3 regions were the most polymorphic; however, some substitutions were observed in all regions. In addition, length differences in the V1, V2, and V5 regions were seen, and these did not result in frameshift mutations.

Biological characterization of parental and chimeric viruses

In order to study the biological effects of the different envelope genes, in isolation from the rest of the λ genome, we transferred both the gp120- and the gp160-encoding regions into the molecular clone pHXB2-MCS (McKeating *et al.,* 1993a). The cloning sites transferred the gp120 region from amino acid 38 (7 amino acids after the end of the signal peptide) to 6 amino acids prior to the gp120/gp41 cleavage signal. The same amino-terminal cloning site was used to clone the gp160 gene, together with the first 17 amino acids of *nef.* The fragments cloned were generated by PCR and therefore replicate clones were analyzed in order to reduce the possibility of PCR-induced error. Transfection of HXB2, λ, and chimeric clones into both HeLa and HeLa–CD4 cells gave rise to virions that were capable of replicating in PBMC (Table 1). Transfected HeLa–CD4 cells were screened for virus replication both by assays of extracellular p24 antigen and by the detection of intracellular p24 antigen producing foci. All clones gave rise to multinucleated foci (Table 1). We previously reported that λ clone 33 was nonviable (Frederiksson *et al.,* 1991); however, sequence analysis showed that this clone has a full gp120 open reading frame (Fig. 1). Moreover, the chimeric HXB2.gp120 clone expressing this envelope was viable (Table 1), suggesting that any defect(s) is located elsewhere in the genome. Transfected HeLa cells were cocultured with PBMC for 24 hr. All viruses replicated in PBMC, resulting in comparable levels of p24 antigen (Table 1). Production of infectious virus, as measured by the ID_{50}, was reduced for all HXB2.gp120 chimeric viruses compared to the λ clones. However, both HXB2 and the gp160 chimeras (λ2 and 31) replicated poorly in PBMC, resulting in low levels of infectious virus (Table 1). These

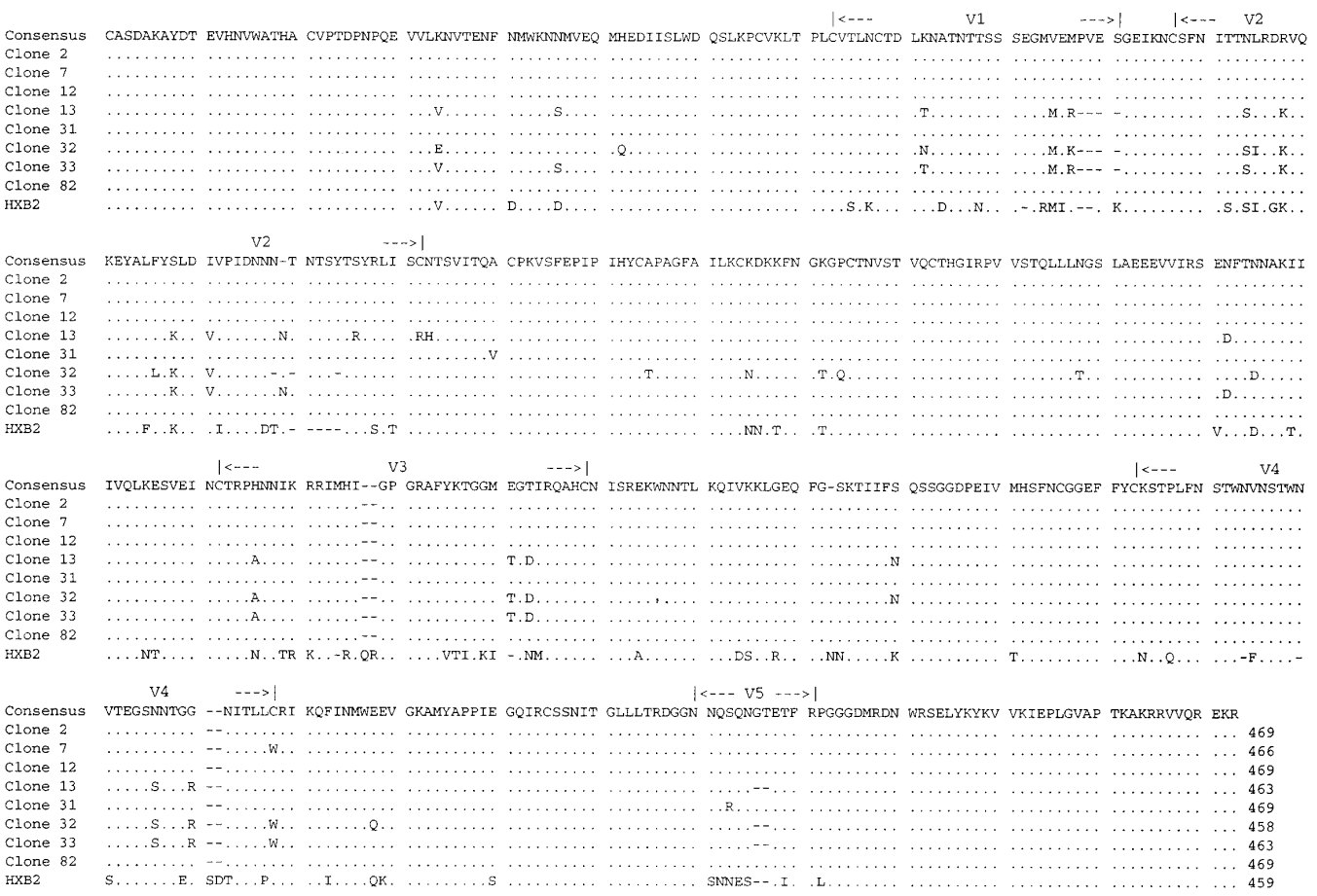

FIG. 1. Peptide translation of gp120 sequences from KI4803 λ clones. The consensus is shown above the alignment, identity is indicated by a dot, departures from the consensus are indicated by the single letter amino acid codes, and "-" indicates that an amino acid is missing. The sequence of the HXB2 gp120 is indicated for comparative purposes.

data suggest that the envelope glycoprotein is one of the determinants of replication rate in PBMC cultures.

We have previously reported differences in the cell tropism of virus derived from the λ clones (Tan *et al.,* 1993). We therefore tested the ability of a number of viruses chimeric for gp120 to replicate in a range of T-cell lines. Infected PBMC were cocultivated with MT2, SupT1, U937, and CEM cells in the absence of IL-2 and were monitored both for cytopathic effect and for the production of p24 antigen (Table 2A). Syncytia were observed in both MT2 and SupT1 cells with all of the clones tested; similarly, p24 antigen was detected in all of these cultures. In contrast, only HXB2, λ32, and HXB2.gp120λ32 were able to replicate in U937 and CEM cells (Table 2A). These observations demonstrate that the ability of a virus to replicate in both U937 and CEM cells is determined by gp120 and that the gp120 chimeric viruses are phenotypically similar to the λ clones from which they derive with respect to their patterns of cell tropism. Extracellular virus from transfected HeLa–CD4 cells was assessed for its ability to infect both PBMC and the T-cell lines C8166 and SupT1 (Table 2B). All of the chimeric viruses replicated in the PBMC and SupT1 cells, resulting in infectious titers greater than 75 ID_{50}/ml. However, HXB2 replicated preferentially in C8166 cells, yielding a titer of 380 ID_{50}/ml compared to 50 and <5 ID_{50}/ml in SupT1 and PBMC cultures, respectively.

Epitopes capable of inducing neutralizing antibodies have been mapped to both gp120 and gp41 (reviewed in Steimer *et al.,* 1991). In order to study the relative contribution of gp120 epitopes to the overall immune response we compared the sensitivity of HXB2, primary λ, and a number of chimeric viruses to neutralization by a panel of sera from HIV-infected individuals (Table 3A). Since HXB2 failed to produce detectable levels of extracellular virus in the PBMC cultures, we transfected H9 cells to obtain HXB2 sufficient for neutralization studies. We (data not shown) and others (Wrin *et al.,* 1995) have demonstrated that the sensitivity of a virus to neutralization is the same irrespective of the cell used for propagation. This is in contrast to that reported by Sawyer and colleagues (1994) who stated that several passages of a virus through a cell line were required to observe differences in neutralization sensitivity suggesting viral adaptation. However, we only propagated HXB2 in H9 cells for 7 days. Seven of the eleven sera tested were able to

TABLE 1

Transfection of HXB2, λ Clones, and gp120 and gp160 Chimeras

Clone	Cytopathicity in HeLa–CD4	p24 antigen (ng/ml) Hela[a]	p24 antigen (ng/ml) PBMC[b]	ID_{50} PBMC[c]
λCL2	+	ud	2.4	280
λCL13	+	ud	1.8	75
λCL31	++	ud	2.8	440
λCL32	++	ud	3.1	310
λCL82	+	ND	2.1	280
HXB2	++	1.1	1.6	<5
HXB2.gp120CL2	+	1.1	3.2	125
HXB2.gp120CL13	+	4.5	2.8	75
HXB2.gp120CL31	++	4.8	3.4	185
HXB2.gp120CL32	++	4.8	3.4	ND
HXB2.gp120CL33	+	3.6	3.1	ND
HXB2.gp120CL82	+	5.0	4.3	100
HXB2.gp160CL2	+	1.0	1.6	<5
HXB2.gp160CL31	+	0.9	1.4	<5

Note. HXB2, λ, and chimeric plasmid DNA were transfected into HeLa and HeLa–CD4 cells and monitored for signs of infection. Soluble p24 antigen was measured in the HeLa[a] cells 48 hr posttransfection. Transfected HeLa cells were cocultuivated with PBMC for 24 hr and the nonadherent PBMC maintained for 21 days in culture. PBMC cultures were assessed both for soluble p24 antigen[b] and infectious virus[c] (ID_{50}/75 μl) 7 days postinfection. ud, undetectable levels of p24 antigen; ND, not done.

neutralize HXB2 and two or more of the chimeric viruses; in contrast, only one serum (no. 3) neutralized more than one of the λ clones. The ability of the sera to neutralize the gp120 chimeric viruses could be mediated via epitopes outside gp120; alternatively, the interaction of λ.gp120 with HXB2 gp41 could result in a novel conformation(s) unique to the chimeric molecule. In order to test the latter hypothesis we measured the ability of a number of gp120-specific, conformation-dependent ligands to neutralize HXB2, λ, and chimeric viruses (Table 3B). sCD4, three mAbs mapping to the discontinuous CD4 b.s. (1.5e, 2.1H, and 39.13g) (Cordell *et al.,* 1991; Ho *et al.,* 1991), and two mAbs mapping to linear determinants within V2 (10/76b, 12b) (McKeating *et al.,* 1993b; Shotton *et al.,* 1995) together with mAb 268-D, specific for a linear epitope within V3 (Gorny *et al.,* 1989), at a final concentration of 20 μg/ml, failed to neutralize any of the viruses expressing primary glycoproteins, whereas all of these ligands, with the exception of 268-D, were able to neutralize HXB2. In contrast, mAb 2G12, mapping to a discontinuous epitope distinct from the CD4 binding site (Buchacher *et al.,* 1994), was able to neutralize all of the viruses. A control mAb, 2F5, specific for a linear epitope in gp41 (Muster *et al.,* 1993; Buchacher *et al.,* 1994; Conley *et al.,* 1994b) neutralized HXB2, all of the chimeric viruses, and two of the four λ viruses (Table 3B). These data fail to show any differences in the gp120 conformation between the λ and chimeric viruses.

The inability of the gp120-specific mAbs to neutralize both the λ and the chimeric viruses could be attributable to antigenic variation. To test this possibility we followed the ability of the mAbs to bind to detergent-solubilized gp120 obtained from the same viral stocks as used for the neutralization studies. Levels of gp120 present in the viral lysates were quantified in an ELISA using a pool of sera from HIV-infected individuals as the detecting antibody (Table 4). All of the viral lysates bound sCD4 with comparable efficiencies. MAbs, 39.13g and 1.5e, mapping to the CD4 b.s., bound to λ31, 32, and HXB2, but showed no recognition of λ2 and 13. In addition, both λ2 and 13 bound with low OD values to mAb 2.1h, suggesting that this group of mAbs recognizes distinct epitopes within the CD4 binding region. In contrast, mAbs 268-D and 2G12 bound all the λ proteins, though with reduced OD values for both λ2 and 13. HXB2 failed

TABLE 2A

Comparative Replication of HXB2, λ and gp120 Chimeric Viruses in T-Cell Lines: Cell–Cell Coculture

Clone	p24 (ng/ml) 3 days p.i. in MT-2	SupT1	U937-2	CEM	p24 (ng/ml) 8 days p.i. in MT-2	SupT1	U937-2	CEM
λCL2	24.2	6.0	ud	ud	>50.0	>50.0	ud	ud
λCL13	48.4	42.5	ud	ud	>50.0	>50.0	ud	ud
λCL31	18.2	3.0	ud	ud	>50.0	>50.0	ud	ud
λCL32	45.6	22.5	15.3	15.4	>50.0	>50.0	>50.0	>50.0
λCL82	24.9	11.2	ud	ud	>50.0	>50.0	ud	ud
HXB2	46.3	48.5	6.5	4.5	>50.0	>50.0	45.2	39.5
HXB2.gp120CL2	21.4	12.5	ud	ud	>50.0	>50.0	ud	ud
HXB2.gp120CL13	46.3	39.5	ud	ud	>50.0	>50.0	ud	ud
HXB2.gp120CL31	40.5	46.7	ud	ud	>50.0	>50.0	ud	ud
HXB2.gp120CL32	44.2	46.3	12.4	6.5	>50.0	>50.0	>50.0	>50.0
HXB2.gp120CL82	39.5	42.1	ud	ud	>50.0	>50.0	ud	ud

Note. ud, undetectable levels of p24 antigen (<75 pg/ml).

TABLE 2B

Comparative Replication of HXB2 and gp120 Chimeric Viruses in T-Cell Lines: Cell-Free Infection

Clone	Infectious titer (ID_{50})/0.075 ml for cells		
	C8166	SupT1	PBMC
HXB2	380	50	<5
HXB2.gp120CL2	<5	120	80
HXB2.gp120CL13	15	140	75
HXB2.gp120CL31	<5	135	85
HXB2.gp120CL32	18	170	110
HXB2.gp120CL82	10	180	120

to bind the V3 mAb 268-D. Only one of the λ proteins, λ31, was found to bind the V2 mAb 12b, suggesting antigenic variation within the V2 region among the λ clones. We were able to detect the binding of mAb 2G12 to the surface of all infected PBMC cultures by FACS analysis, whereas we failed to detect binding of any of the other mAbs to the infected cell surface (data not shown).

In order to assess whether gp120-gp41 association differed between the λ and gp120 chimeric viruses we measured the amount of gp120 shed from the surface of the infected PBMC cultures in the presence and absence of sCD4 (at 20 μg/ml) at both 4 and 37° (Table 5). No soluble gp120 was detected at 4°; however, gp120 was detected from all cultures at 37° in the presence of sCD4. Some variation was observed in the amount of gp120 spontaneously released at 37°; however, the chimeric viruses did not behave differently from HXB2. Soluble CD4-induced shedding of gp120 for both λ and chimeric viruses was not appreciably different, with a mean increase for the λ clones of 1.7 and for the chimeric viruses of 1.8 (not including λ13, λ82, and HXB2.CLλ2, for which no gp120 was detected in the absence of sCD4) (Table 5).

DISCUSSION

These experiments demonstrate that it is possible to construct chimeric viruses expressing primary gp120 envelope proteins. The viability of such viruses is consistent with the functional conservation of the gp120 amino- and carboxy-termini, which are reported to be essential for association with the transmembrane glycoprotein gp41 (Helseth *et al.,* 1991; Schulz *et al.,* 1992). No variability was detected in the amino-terminus (aa 1–31) and only five substitutions were detected in the carboxyl C5 domain (aa 410–465 of HXB2, Fig. 1). This conclusion is further supported by the viability of a series of HXB2 clones chimeric for gp120 regions derived from divergent primary viruses from clades A–G (McKeating, unpublished observation). Transfer of the primary gp120 region to HXB2 conferred the same pattern of cell tropism as demonstrated for the original primary virus molecular clone(s) (Table 2). The V3 region has been reported to be one of the determinants of T-cell tropism (De-Jong *et al.,* 1992); however, we found that λ32, which was able to infect both CEM and U937 cells, had the same V3 coding sequence as λ clones 13 and 33, which were unable to infect these cell lines. However, λ clone 32 had a unique deletion in the V2 region at a position previously reported to be associated with a switch from NSI to SI phenotype (Schuitemaker *et al.,* 1992; Groenink *et al.,* 1993), in addition to several unique substitutions in the C1, V1, C2, C3, and C4 regions (Fig. 1). We (Palmer *et al.,* 1996) and others (Wang *et al.,* 1995) have analyzed V2 length polymorphism in NSI and SI viruses obtained from the same individuals over time and find no correlation between V2 length and tropism for MT-2 cells. Stamatos and Cheng-Mayer (1993) reported that a change at position 282 (N/D) within the C2 region was important for the ability of HXB2 to replicate efficiently and induce syncytia in the Hut-78 cell line. It is interesting to note that λ clone 32 encoded an aspartic acid (D) at this

TABLE 3A

Neutralization of HXB2, **λ**, and Chimeric Viruses

Virus	ID_{50}	Neutralization with HIV^+ human sera										
		1	2	3	4	5	6	7	8	9	10	11
λCL2	15	+	−	+	−	−	−	−	−	−	−	−
λCL13	9	−	−	−	−	−	−	−	−	−	−	−
λCL31	17	−	−	−	−	−	−	−	−	−	−	−
λCL82	8	−	−	+	−	−	−	−	−	−	−	−
HXB2	25	+	−	+	+	+	−	+	−	+	−	+
HXB2.gp120 CL2	15	+	−	+	+	+	−	+	−	+	−	+
HXB2.gp120 CL13	20	+	−	+	+	+	−	+	−	−	−	+
HXB2.gp120 CL31	5	+	−	+	−	+	−	+	−	−	−	+
HXB2.gp120 CL82	15	+	−	+	+	+	−	+	−	+	−	−

Note. Human sera were used at a final concentration of 1/40. +, >90% inhibition of p24 antigen production in all three replicate wells; −, no reduction in p24 production in all replicate wells.

TABLE 3B

Neutralization of HXB2, λ, and Chimeric Viruses

Virus	ID_{50}	Neutralization with ligands[a]								
	Ligand:	268-D	1.5e	2.1H	39.13g	2F5	2G12	10/76b	12b	sCD4
	Epitope:	V3	CD4bs	CD4bs	CD4bs	gp411	gp120	V2	V2	
λCL2	11	−	−	−	−	+	+	−	−	−
λCL13	15	−	−	−	−	−	+	−	−	−
λCL31	18	−	−	−	−	+	+	−	−	−
λCL82	11	−	−	−	−	−	+	−	−	−
HXB2	25	−	+	+	+	+	+	+	+	+
HXB2.pg120CL2	5	−	−	−	−	+	+	−	−	−
HXB2.gp120CL13	15	−	−	−	−	+	+	−	−	−
HXB2.gp120CL31	18	−	−	−	−	+	+	−	−	−
HXB2.gp120CL82	20	−	−	−	−	+	+	−	−	−

[a] All mAbs and sCD4 were used at a final concentration of 20 μg/ml.

position and virus derived from this clone exhibited the broadest tropism to replicate in all cell lines tested (Tan *et al.,* 1993).

We noted that equivalent amounts of p24 antigen were produced in all infected PBMC cultures; furthermore, cultures infected with virus derived from both λ and chimeric constructs resulted in similar levels of infectious extracellular virus (ID_{50}) (Table 1). In contrast, cultures infected with either HXB2 or the gp160 chimeras failed to produce detectable levels of extracellular infectious virus. These data confirm earlier observations of the poor correlation between levels of extracellular p24 antigen and infectious titer (McKeating *et al.,* 1990). Replacement of the gp120 region of HXB2 with that of a primary virus enabled the virus to replicate more efficiently in PBMC. However, the gp160 chimeras replicated poorly; one explanation for this result is that other elements (tat, rev, nef, RRE) contained within the transferred fragment may be disrupted. Cell-free infection experiments showed that the gp120 chimeras replicated to a higher titer than HXB2 in the Sup T1 cell line (Table 2B). It is of interest to note that this cell line expresses a high level of CD4 (mean fluorescence intensity, MFI, of 714) compared to C8166 cells (MFI 56) (data not shown). These observations are in agreement with those reported by Kabat and colleagues (1994) who demonstrated a positive correlation between the replication of primary virus isolates and CD4 receptor expression levels in target cells. This correlation was not observed for T-cell-adapted viruses. These data suggest that primary viruses differ from T-cell-adapted (HXB2) envelope glycoproteins in their interaction with the surface of PBMC and of T-cell lines, whereby the levels of cell surface CD4 may be rate-limiting for their infection process.

Moore and colleagues (1995) reported that primary viruses, propagated in PBMC cultures, are relatively resistant to neutralization by mAbs and human sera compared to T-cell-line-adapted viruses. We were interested in determining if gp120 chimeras, possessing a constant HXB2 gp41, showed altered sensitivity to neutralization compared to the parental viruses. Our results clearly indicate that the chimeric viruses were as sensitive as HXB2 to neutralization by polyclonal human sera (Table 3B). Since the envelope glycoprotein has been identified as the major antigen responsible for the induction of neutralizing antibodies, one interpretation of these data is that gp41-specific antibodies constitute a substantial component of the cross-neutralizing activity present in

TABLE 4

Reactivity of Ligands with Soluble Viral gp120/gp160

Clone	Bound ligand							
	268-D	1.5e	2.1H	39.13g	2G12	12b	sCD4	HIV + pool
λCL2	+	−	+	−	+	−	++	+++
λCL13	+	−	+	−	++	−	++	++
λCL31	++	+	++	++	+++	++	++	++
λCL32	++	+	++	+	+++	−	++	+++
HXB2	−	+	++	++	+++	+++	++	+++

Note. Optical density (450 nm): −, <0.10; +, 0.10–0.49; ++, 0.50–0.99; +++, >1.00.

TABLE 5

Ability of sCD4 to Induce gp120 Dissociation from the Surface of Infected PBMC Cultures

Clone	Soluble gp120 (ng/ml) from 2×10^6 infected PBMC			
	4°	4° + sCD4	37°	37° + sCD4
λCL2	ud	ud	12.0	21.0
λCL13	ud	ud	ud	10.5
λCL31	ud	ud	7.5	18.4
λCL32	ud	ud	7.0	14.3
λCL82	ud	ud	ud	14.8
HXB2	ud	ud	8.4	18.5
HXB2.gp120CL2	ud	ud	ud	7.4
HXB2.gp120CL13	ud	ud	9.2	23.0
HXB2.gp120CL31	ud	ud	13.0	21.5
HXB2.gp120CL32	ud	ud	14.5	25.0
HXB2.gp120CL82	ud	ud	20.1	25.8

Note. ud, undetectable levels, i.e., <200 pg/ml.

human sera. A conserved neutralization epitope within gp41 (aa ELDKWA) has been identified by a human mAb 2F5, which has been reported to cross-neutralize several primary isolates within clade B (Muster *et al.,* 1993; Buchacher *et al.,* 1994; Conley *et al.,* 1994a; Kattinger, 1994). Preliminary studies comparing HXB2 with a number of HXB2 chimeras expressing divergent primary gp120 proteins show that all viruses are equally sensitive to neutralization by sera from individuals infected with clade B viruses (data not shown), further suggesting the importance of conserved gp41 epitopes. This interpretation of the data is further supported by the ability of some human sera to neutralize viruses of diverse origin, suggesting conservation of epitopes among viruses of different subtypes. Since the gp41 region of the envelope glycoprotein is more conserved than gp120 it constitutes an ideal target for such antibodies.

Alternatively, primary envelope gp120 conformation may be affected by association with the T-cell-adapted HXB2 gp41 moiety, resulting in altered sensitivity to neutralization. However, HXB2 chimeras expressing MN and SF-2 gp120 proteins exhibit the same pattern and sensitivity to neutralization by gp120-specific mAbs as the parental viruses (data not shown), arguing against altered gp120 conformation in these chimeric viruses. Since the majority of gp120-specific mAbs fails to neutralize primary viruses, such a direct comparison is more difficult. We (Klasse *et al.,* 1993) and others (Reitz *et al.,* 1988; Wilson *et al.,* 1990; Thali *et al.,* 1994) reported that mutation of residue 582 (Ala–Thr) in the gp41 domain of HXB2 resulted in a virus which was resistant to neutralization by some antibodies specific for the CD4 binding site, supporting the hypothesis that gp120 conformation can be modulated by amino acid changes in gp41. However, position 582 (Ala) and the surrounding residues were conserved between HXB2 and the λ clones (data not shown). Furthermore, mAbs specific for the CD4 b.s. (1.5e, 2.1h, and 39.13g) were unable to neutralize viruses derived from either λ or gp120.HXB2 clones, suggesting that the presentation of these epitopes was not altered in the chimeric viruses (Table 3B). Furthermore, one gp120-specific mAb, 2G12, recognizing a conformational epitope independent of the CD4 binding site (Buchacher *et al.,* 1994; Trkola *et al.,* 1996), neutralized both λ and chimeric viruses with similar efficiencies (endpoint neutralization titers of 3–8 μg/ml) (data not shown). Sullivan and colleagues (1995) reported an association between the ability of sCD4 to neutralize and its ability to induce dissociation of gp120–gp41 for primary viruses. However, we found that sCD4 was able to induce gp120–gp41 dissociation from both λ- and λgp120.HXB2-infected cells, but was unable to neutralize cell-free virus at the highest concentration tested (20 μg/ml) (Tables 3 and 5).

The effect of antigenic variation on the sensitivity of the viruses to neutralization was tested by following the ability of antibodies to bind to detergent-solubilized viral gp120. The V3 mAb 268D was able to bind all the λ gp120 proteins with an affinity close to that for the MN protein. However, this mAb failed to neutralize any of the λ or chimeric viruses despite its ability to neutralize the MN isolate at a concentration of 0.5 μg/ml (data not shown). These data are in agreement with recent observations suggesting that the V3 region may not be well exposed on primary virion glycoproteins and may therefore not constitute the principal neutralization target (Bou-Habib *et al.,* 1995; Stamatatos and Cheng-Mayer, 1995). In contrast, antigenic variation was observed in the CD4 binding site such that clones 2 and 13 failed to bind mAbs (1.5e, 39.13g) specific for epitopes overlapping this region. However, clones 31 and 32 bound these mAbs with an affinity close to that of HXB2, but were resistant to neutralization. These data suggest that the lack of neutralization of the λ viruses by gp120-specific antibodies may be partially explained by antigenic variation. However, some of the epitopes specific for these ligands have been shown to be present in the solubilized viral protein. Hence, the affinity of a mAb for soluble monomeric gp120 does not correlate with its ability to neutralize, in agreement with previous reports (Sullivan *et al.,* 1995; Sattentau *et al.,* 1994). We therefore attempted to measure the ability of the mAbs, used in the neutralization assays, to bind to the oligomeric envelope surface of infected PBMC cultures. However, with the exception of 2G12, we failed to detect any cell surface binding, and this result correlated with the ability of 2G12 to neutralize all viruses tested. Similarly, Sullivan and colleagues (1995) reported that neutralization of virus by antibodies or sCD4 could be predicted by assays measuring the binding of these ligands to the oligomeric envelope glycoprotein complex. Consequently, it will be important to study the immunogenicity of recombinant

oligomeric gp140 envelope proteins for their ability to induce antibodies capable of cross-neutralizing primary virus isolates (Earl *et al.,* 1994).

In summary, HXB2 and chimeric viruses expressing primary gp120 proteins were neutralized by polyclonal human sera. In contrast, the same chimeras were resistant to neutralization by sCD4 and the majority of gp120-specific mAbs. Clearly, the chimeras behave more like the primary λ viruses with respect to cell tropism, gp120–gp41 association, and their relative resistance to neutralization by gp120-specific ligands. However, they behave more like HXB2 with respect to their neutralization by polyclonal human sera. These data suggest that the essential properties which define a "primary virus oligomer" are defined by the gp120 region and that cross-reactive neutralizing antibodies present in human sera may be targeted to epitopes in gp41.

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